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ADP-ribosylation factors: a family of ~20-kDa guanine nucleotide-binding proteins that activate cholera toxin

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Abstract

ADP-ribosylation factors (ARFs) comprise a family of ~20 kDa guanine nucleotide-binding proteins that were discovered as one of several cofactors required in cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$, the guanine nucleotide-binding protein responsible for stimulation of adenylyl cyclase, and was subsequently found to enhance all cholera toxin-catalyzed reactions and to directly interact with, and activate the toxin. ARF is dependent on GTP or its analogues for activity, binds GTP with high affinity in the presence of dimyristoylphosphatidylcholine/cholate and contains consensus sequences for GTP-binding and hydrolysis. Six mammalian family members have been identified which have been classified into three groups (Class I, II, and III) based on size, deduced amino acid sequence identity, phylogenetic analysis and gene structure. ARFs are ubiquitous among eukaryotes, with a deduced amino acid sequence that is highly conserved across diverse species. They have recently been shown to associate with phospholipid and Golgi membranes in a GTP-dependent manner and are involved in regulating vesicular transport. (Mol Cell Biochem **138**: 157–166, 1994)

Key words: cholera toxin, adenylyl cyclase, ADP-ribosylation factors, guanine nucleotide-binding (G) proteins, vesicular trafficking

Abbreviations: ARF – ADP-ribosylation factor; sARF I and sARF II – soluble ADP-ribosylation factors purified from bovine brain; mARF – purified membrane-associated ARF; hARF – human ARF; bARF – bovine ARF; yARF – yeast ARF; rARF – bacterially-expressed recombinant ARF; gARF – *Giardia* ARF; dARF – *Drosophila* ARF; G protein – guanine nucleotide-binding protein; G_s – G protein responsible for stimulation of adenylyl cyclase; GTPγS – guanosine-5′-O-(3-thio-triphosphate); CTA1 – cholera toxin A1 subunit; DMPC – dimyristoylphosphatidylcholine; SDS – sodium dodecyl sulfate

Introduction

ADP-ribosylation factors are ~20 kDa guanine nucleotide-binding proteins that were initially discovered because they are cofactors for cholera toxin's enzymatic activities. Cholera toxin is a hetero-oligomeric protein secreted by *Vibrio cholerae* and composed of one A and five B subunits, the latter forming the cell membrane binding domain [1, 2]. The active A1 peptide (CTA1) is generated after reduction of a disulfide bond that links it to the A2 polypeptide [3, 4]. CTA1 is responsible for cholera toxin's enzymatic activities [5]. These include (i) NAD hydrolysis to ADP-ribose and nicotinamide [6]; (ii) transfer of ADP-ribose to free arginine and simple guanidino compounds [7, 8]; (iii) ADP-ribosylation of nonspecific proteins, presumably due to available arginine residues which serve as toxin substrates [9]; (iv) auto-ADP-ribosylation of CTA1 [10, 11]; and (v) ADP-ribosylation of $G_{s\alpha}$, the guanine nucleotide-binding protein responsible for stimulation of adenylyl cyclase [12–15]. $G_{s\alpha}$ is the substrate for cholera toxin *in vivo* and its modification by toxin underlies the pathogenesis of cholera by causing stimulation of adenylyl cyclase in intestinal mucosal cells [16, 17].

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Activation of adenylyl cyclase by cholera toxin was shown to require NAD [18], GTP or analogues [19–22], and certain cellular factors [19]. In membrane preparations, cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ also required GTP or nonhydrolyzable analogs and a cytosolic factor and correlated well with activation of adenylyl cyclase [23]. Two guanine nucleotide-binding sites were involved in this system: one responsible for activation of adenylyl cyclase and a distinct site which supported ADP-ribosylation but not cyclase activity [24].

Various cellular factors that augmented cholera toxin catalyzed ADP-ribosylation of $G_{s\alpha}$ and/or activation of adenylyl cyclase were described [19, 23, 25–35]. These factors, which were isolated from numerous tissues and species, varied in size, biochemical properties, and subcellular localization.

Purification and biochemical characterization of ARF

A membrane-associated ADP-ribosylation factor (mARF) was purified 1800-fold from rabbit liver membranes [32]. Protein stability required detergent throughout the purification. ARF migrated as a doublet on SDS polyacrylamide gel electrophoresis and exhibited a M_r of 21,500 [32]. GTP was required for ARF to stimulate cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ and ARF appeared to act catalytically in the assay [32].

ARF purified from bovine brain membranes bound guanine nucleotides (GTP, GDP, and GTPγS), but not adenine nucleotides [33]. High-affinity binding occurred in the presence of dimyristoylphosphatidylcholine (DMPC), $MgCl_2$, and high ionic strength (0.8 M NaCl). The apparent dissociation constants for GTP and GDP were 90 and 40 nM, respectively. Purified ARF contained approximately equimolar quantities of bound GDP, however no GTPase activity was detected *in vitro*. ARF-GTP was active in supporting cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ whereas ARF-GDP was inactive [33].

ARF also enhanced $G_{s\alpha}$-independent reactions catalyzed by the toxin A1 peptide (CTA1) [34, 35]. These included the hydrolysis of NAD to nicotinamide and ADP-ribose, the auto-ADP-ribosylation of CTA1, and ADP-ribosylation of simple guanidino compounds such as agmatine. In these reactions, ARF required GTP or a nonhydrolyzable analogue, whereas GDP and ATP analogues were ineffective [34, 35]. A model of a guanine nucleotide-binding protein cascade for cholera toxin activation of adenylyl cyclase has been proposed (Fig. 1). ARF, activated by GTP, interacts with, and activates the toxin A1 peptide which, in turn, catalyzes the ADP-ribosylation of $G_{s\alpha}$. In the presence of GTP, modified $G_{s\alpha}$ persistently stimulates the adenylyl cyclase catalytic subunit. In this model, there is no direct interaction between ARF and the toxin substrate.

Kinetic analysis of ARF-stimulated NAD:agmatine ADP-ribosyltransferase activity of CTA1 is consistent with direct interaction between ARF and the toxin subunit [36]. In the absence of detergents, ARF plus GTP lowered the apparent K_m for NAD and agmatine. In the presence of 0.003% SDS, the concentration at which activity was maximal, the apparent K_m values for both substrates were lowered further and the V_{max} was increased [36]. It was concluded that ARF is a direct allosteric activator of the toxin A1 subunit.

Since ARF's ability to stimulate cholera toxin-catalyzed reactions is dependent on GTP or its analogues, conditions optimal for high-affinity binding were determined [37]. Maximal binding of GTP by sARFII was observed in the presence of 3 mM DMPC and 0.2% cholate and required Mg^{2+} but not NaCl [37]. The apparent K_D for GTP was 70 nM [37]. In the presence of low concentrations of SDS, conditions optimal for ARF stimulation of cholera toxin ADP-ribosyltransferase activity, minimal high affinity GTP binding by sARF II was observed [37]. Consistent with the binding data, the EC_{50} for GTP in stimulation of cholera toxin ADP-ribosyltransferase activity was 5 μM in the presence of 0.003% SDS and 50 nM with DMPC/cholate. Thus, ARF stimulates cholera toxin-catalyzed reactions under conditions that support either high-(DMPC/cholate) or low-(SDS) affinity interactions with GTP.

ARF stimulation of cholera toxin reactions, both $G_{s\alpha}$-dependent and $G_{s\alpha}$-independent, was influenced by detergents and phospholipids [35–37]. DMPC was required for sARF plus GTP to stimulate maximally ADP-ribosylation of $G_{s\alpha}$ but not auto-ADP-ribosylation of CTA1, which was somewhat inhibited [35, 37]. Maximal ARF stimulation of NAD: agmatine ADP-ribosyltransferase activity was observed in the presence of nondenaturing concentrations of SDS [36, 37] as was CTA1 auto-ADP-ribosylation [36, 38].

In the presence of GTPγS and SDS, a fraction of ARF formed a complex with CTA1 [38]. Monomeric ARF separated from the complex by gel filtration was incapable of further complex formation. ARF-CTA1 complex stimulated auto-ADP-ribosylation to a greater extent than did the monomeric species, which exhibited clearly different substrate specificities [38]. No ARF-CTA1 complex was formed during incubation with GTPγS and DMPC/cholate. ARF, however, became aggregated under these conditions and was more active in stimulating cholera toxin ADP-ribosylation of added substrates than auto-ADP-ribosylation of CTA1 [38]. These studies may provide a biochemical basis for the observed effects of detergents and phospholipids on substrate specificity.

Six mammalian ARF family members have been identified through molecular cloning (see section on Molecular Characterization of ARF for complete discussion) and each of these has been expressed in bacteria [39–41]. Purified recombinant ARFs (rARFs) each stimulated cholera toxin-

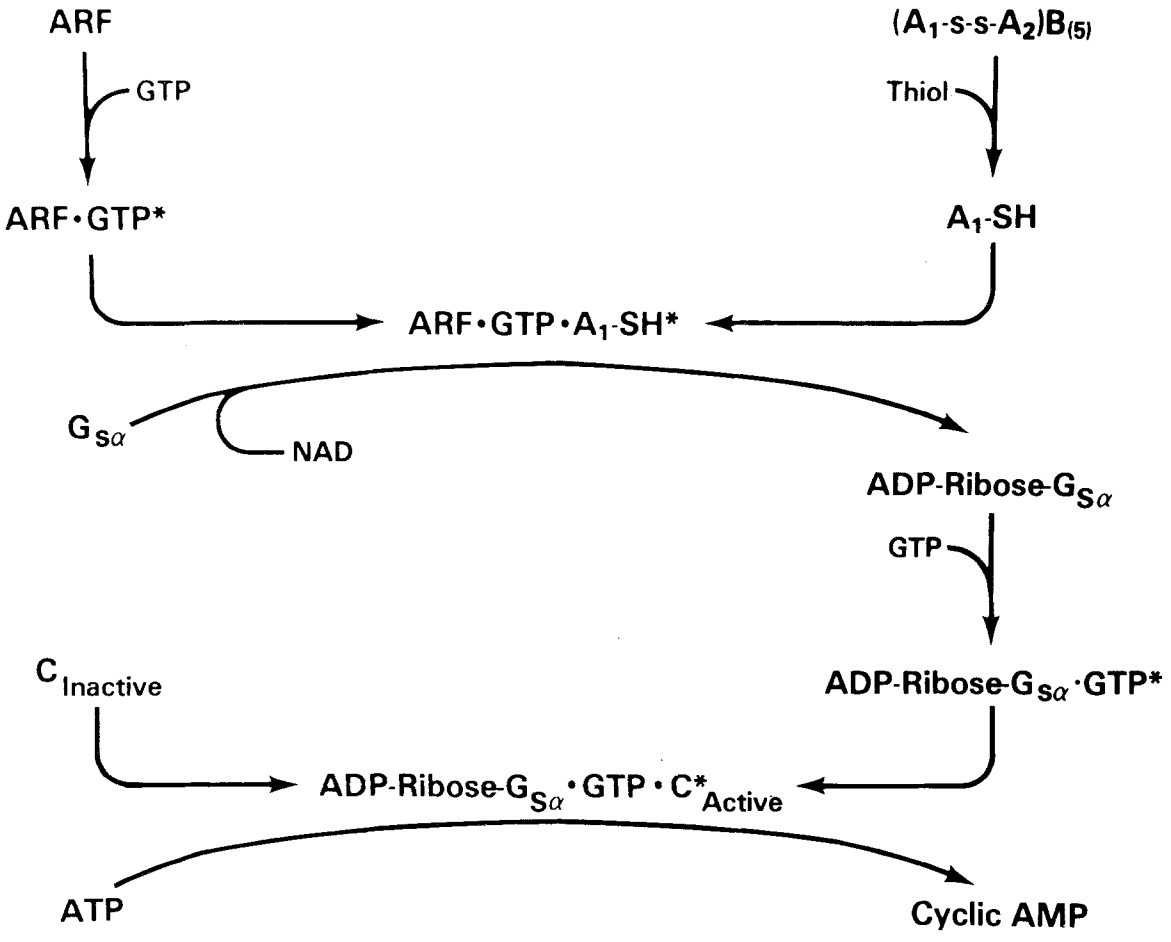

Fig. 1. ARF participation in a guanine nucleotide-binding protein cascade resulting in activation of adenylyl cyclase. Activated ARF (ARF-GTP*) interacts with toxin A1 protein (A_1-SH), generated from holotoxin (A_1-ss-A_2)$B_{(5)}$ by reduction with thiol. Activated toxin catalyzes the transfer of ADP-ribose from NAD to $G_{s\alpha}$. In the presence of GTP, modified $G_{s\alpha}$ persistently stimulates the adenylyl cyclase catalytic subunit (C), generating cyclic AMP from ATP. Data are from Ref. 35.

catalyzed ADP-ribosyltransferase activity, using either $G_{s\alpha}$ or agmatine as substrate. Individual differences were noted, however, in the conditions under which maximal activity was observed. Whereas rARFs 1–5 required DMPC/cholate for activity, rARF 6 was active in the presence of SDS, DMPC/cholate, cholate alone, and in the absence of detergents and lipids [41]. Other differences observed between individual ARFs included sensitivity to high ionic strength, the EC_{50} for GTP, and the requirement for Mg^{2+} in binding guanine nucleotides [39, 41]. Differences in biochemical properties noted during early characterization and purification of factors required for cholera toxin activation may be related to such differences among individual ARFs.

ARF developmental expression

Expression of ARF proteins differs with developmental stage in some tissues. sARF I and sARF II (two forms purified from bovine brain) expression was studied in post-natal rat brain cytosol [42]. The level of immunoreactive sARF II relative to sARF I increased between postnatal days 2 and 10. The level of ARF 3 mRNA increased with age while there were simultaneous decreases in levels of ARF 2 and 4 mRNAs and ARF 1, 5, and 6 mRNAs were unchanged [42].

Tissue-specific differences in ARF 4 mRNA during development have also been observed [43]. A short form of ARF 4 mRNA (~1.1 kb) was found only in the testis of several species, along with the ubiquitous 1.8-kb mRNA. The 1.1 kb-species resulted from use of the first of three putative polyadenylation signals in the 3′-untranslated region [43]. During development of rat testis, the 1.1-kb mRNA did not appear until the 35th postnatal day, suggesting that appearance of the shorter message may be associated with a late stage of spermatogenesis [43], a suggestion corroborated by *in situ* hybridization.

Molecular characterization of ARF

There are at least six mammalian ARFs based on isolation and sequencing of cDNA clones [40, 44–49]. ARF genes have also been cloned from yeast (yARFs 1 and 2) [44, 50], *Drosophila melanogaster* (dARF 1) [51], and the protozoan *Giardia lamblia* (gARF) [52]. With the exception of ARF 2, which has not been found in human or monkey [42, 48, 53], the individual ARFs appear to be highly conserved across species (Table 1). For example, the human and bovine deduced amino acid and coding region nucleotide sequences for ARF 1 are 100% and 91% identical, respectively [45]. The *Giardia* ARF gene encodes a protein of 191 amino acids 63–70% identical to mammalian ARFs, remarkable conservation considering that the divergence of this protozoan from other eukaryotes has been estimated to be ~1.5 billion years ago [52].

Table 1 highlights the conservation of nucleotide and deduced amino acid sequences among individual ARF family members. Based on these comparisons, the mammalian ARFs have been assigned to three classes. Class I ARFs (ARFs 1, 2, and 3) are 95–96% identical to each other in deduced amino acid sequence, 79–80% identical to Class II and 68–69% identical to Class III ARFs. Class I ARFs are 181 amino acids in length. Class II is composed of ARFs 4 and 5 which contain 180 amino acids and are 90% identical to each other. The sole known member of Class III is ARF 6, which has only 175 amino acids and is 64–69% identical to other mammalian ARFs. Sequence differences among Classes I, II, and III are located primarily in the amino terminal region and carboxyl half of the proteins (Fig. 2).

The classification of ARF proteins is corroborated by an evaluation of evolutionary relationships among ARF family members [48]. Phylogenetic analysis was performed using coding region nucleotide sequences of the individual ARFs and is shown in Fig. 3. Branch points, indicating evolutionary divergence, correspond to each class of mammalian ARF and yARF 1.

The ARFs represent distinct gene products. Each of the Class I ARF genes has been cloned and its structure determined (Fig. 4) [54–56]. Each of the genes contains five exons and four introns. The first exon is untranslated and translation initiation is located in exon 2. The consensus sequences for GTP binding and hydrolysis, which are identical in all mammalian ARFs, are encoded in separate exons. The sequence GLDAAGK, the putative phosphate binding loop, is encoded in exon 2 and DVGG, which is likely involved in Mg^{2+} coordination, in exon 3. The sequence encoding NKQD (important in guanine ring-binding specificity) is separated by intron 4 into exons 4 and 5 between the codons for glutamine and aspartate. Exon/intron boundaries occur at identical sites within the coding regions of all three genes (Fig. 4). Each of the 5′-untranslated regions contains multiple transcription initiation sites, multiple potential Sp1 binding sites, and no TATA box, similar to promoters of housekeeping genes [54–56].

A *Drosophila* ARF gene, dARF 1, was categorized as Class I based on a high degree of homology with mammalian ARFs 1–3 [51]. Two introns in the *Drosophila* gene contained splice sites very similar to the exon/intron boundaries in the mammalian Class I genes [51]. Gene structure in at least Class I ARFs, thus appears to be conserved across rather distantly related species.

The promoters of ARFs 2 and 3 both have regions that may be important for regulation [56, 57]. The ARF 2 promoter contains six inverted CCAAT motifs unlike those of ARFs 1 and 3 [54–56] and the functional regulatory region of the promoter includes a region containing one of these motifs. Whether this region is important in the species-specific and developmentally regulated expression patterns noted previously is an intriguing speculation, which requires further investigation. The ARF 3 promoter region contains a palindromic sequence important for transcription activity, which binds a protein from nuclear extracts [57]. A mutated palindromic sequence, which failed to compete for binding of the nuclear factor, had reduced transcription activity when linked

Table 1. Percentage identity of ARF deduced amino acid sequences and coding region nucleotide sequences

	hARF1	bARF1	bARF2	hARF3	hARF4	hARF5	hARF6	yARF1	gARF
hARF1	–	100	96	96	80	80	68	77	70
bARF1	91	–	96	96	80	80	68	77	70
bARF2	79	80	–	95	80	80	69	77	70
hARF3	84	84	80	–	79	79	68	76	69
hARF4	67	68	68	71	–	90	64	72	69
hARF5	75	73	71	73	77	–	64	69	69
hARF6	68	69	64	66	60	65	–	65	63
yARF1	64	66	66	65	67	64	60	–	62
gARF	65	67	62	64	61	66	62	67	–

Comparison of amino acid and nucleotide sequences of ARF family proteins. Percentage identity of deduced amino acid sequences is above the diagonal and of coding region nucleotide sequences below. See Fig. 2 for explanation of abbreviations and references. Data are from Refs. 48, 52.

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                    10         20         30         40         50         60         70
hARF1          1    MGnifanLFk gLFGKKEMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IsFTVWDVGG
bARF2          1    MGnvfekLFk sLFGKKEMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IsFTVWDVGG
hARF3          1    MGnifgnLlk sLiGKKEMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IsFTVWDVGG
hARF4          1    MGltiSsLFs rLFGKKqMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IcFTVWDVGG
hARF5          1    MGltvSaLFs riFGKKqMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IcFTVWDVGG
hARF6          1    MGkvlSk     iFGnKEMRI LMlGLDAAGK TTILYKLKLG qsVTTIPTvG FNVETVtYKN vkFnVWDVGG
yarf1          1    MGlfaSkLFs nLFGnKEMRI LMVGLDgAGK TTvLYKLKLG EviTTIPTIG FNVETVqYKN IsFTVWDVGG
yarf2          1    MGlyaSkLFs nLFGnKEMRI LMVGLDgAGK TTvLYKLKLG EviTTIPTIG FNVETVqYKN IsFTVWDVGG
gARF           1    MGqgaSkiFg kLFsKKEvRI LMVGLDAAGK TTILYKLmLG EvVTTvPTIG FNVETVEYKN InFTVWDVGG
CONSENSUS           MG              K  RI LM GLD AGK TT LYKL LG    TT PT G FNVETV YKN   F VWDVGG

                    80         90        100        110        120        130        140
hARF1         71    QDkIRPLWRH YfQNTQGLIF VVDSNDRE  Rv nEAReELmRM LaEDELRDAV LLVFANKQDL PnAMnaaEIT
bARF2         71    QDkIRPLWRH YfQNTQGLIF VVDSNDRE  Rv nEAReELtRM LaEDELRDAV LLVFvNKQDL PnAMnaaEIT
hARF3         71    QDkIRPLWRH YfQNTQGLIF VVDSNDRE  Rv nEAReELmRM LaEDELRDAV LLVFANKQDL PnAMnaaEIT
hARF4         71    QDrIRPLWkH YfQNTQGLIF VVDSNDRE  Ri qEvadELqkM LlvDELRDAV LLlFANKQDL PnAMaisEmT
hARF5         71    QDkIRPLWRH YfQNTQGLIF VVDSNDRE  Rv qEsadELqkM LqEDELRDAV LLVFANKQDm PnAMpvsElT
hARF6         67    QDkIRPLWRH YytgTQGLIF VVDcaDRd  Ri dEARqELhRi indrEmRDAi iLiFANKQDL PdAMkphEIq
yarf1         71    QDrIRsLWRH YyrNTeGvIF VVDSNDRs  Ri gEARevmqRM LnEDELRnAa wLVFANKQDL PeAMsaaEIT
yarf2         71    QDrIRsLWRH YyrNTeGvIF ViDSNDRs  Ri gEARevmqRM LnEDELRnAV wLVFANKQDL PeAMsaaEIT
gARF          71    QDsIRPLWRH YyQNTdaLIy ViDSaDlEpkRi edARnELhtl LgEDELRDAa LLVFANKQDL PkAMsttdlT
CONSENSUS           QD IR LW H Y   T   I  V D  D    R                 E R A   L F NKQD  P AM

                    150        160        170             180
hARF1         141   dKLGLhSLRh RnWYIQATCA TSGdGLYEGL DWL  SNqL   rNqk        181
bARF2         141   dKLGLhSLRq RnWYIQATCA TSGdGLYEGL DWL  SNqL   KNqk        181
hARF3         141   dKLGLhSLRh RnWYIQATCA TSGdGLYEGL DWL  aNqL   KNkk        181
hARF4         141   dKLGLqSLRn RtWYvQATCA TqGtGLYEGL DWL  SNeLs  Kr          180
hARF5         141   dKLGLqhLRs RtWYvQATCA TqGtGLYdGL DWL  SheLs  Kr          180
hARF6         137   eKLGLtriRd RnWYvQpsCA TSGdGLYEGL tWL tSNy    Ks          175
yarf1         141   eKLGLhSiRn RpWfIQATCA TSGeGLYEGL eWL  SNsL   KNst        181
yarf2         141   eKLGLhSiRn RpWfIQsTCA TSGeGLYEGL eWL  SNnL   KNqs        181
gARF          143   erLGLqeLkk RdWYIQpTCA rSGdGLYqGL DWL  SdyifdkKNkkkgkkr   191
CONSENSUS           LGL       R W  Q  CA   G GLY GL  WL
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Fig. 2. Deduced amino acid sequences of ARF proteins. Capital letters indicate amino acids identical in at least six ARFs. CONSENSUS indicates amino acids identical in all the ARFs. Sequences were aligned using Gene Works program and gaps were introduced where needed for optimal alignment. hARF1, 3–6, human ARFs [45, 47, 48]; bARF2, bovine ARF 2 [46]; dARF1, *Drosophila* ARF [51]; yARF1, 2, yeast ARFs 1 and 2 [44, 50]; and gARF, *Giardia* ARF [52]. Data are from Ref. 52.

to a reporter gene. The identity of this factor and its role in regulating ARF expression, and other TATA-less promoters, is unknown.

ARF protein structure

Through sequence comparisons with other GTPases, such as ras, certain functional domains in ARFs have been inferred. All of the ARF gene products contain the consensus sequences for GTP binding and hydrolysis [58]. These are 100% identical among the mammalian ARFs with minimal and usually conservative differences in yeast and *Giardia* ARFs (Fig. 2). The first GTP-binding sequence GLDAAGK, which spans amino acids 24–29 in hARF 1, corresponds to the phosphate-binding loop in the *ras* gene product. Aspartate 26 in ARF corresponds to Glycine 12 in c-Ha-ras [58], which is essential for normal GTP hydrolysis. In ras, replacement of Glycine 12 by almost any other amino acid markedly reduced GTPase activity and promotes oncogenic transformation [59–61]. The presence of aspartate in this position might help to explain the absence of detectable GTPase activity in the ARF proteins. However, arl, an ARF-like *Drosophila* protein, which also has aspartate in this position, has notably higher basal GTPase activity [62].

The second consensus site for GTP-binding is involved in Mg^{2+} ion coordination. This sequence in all of the known ARFs is DVGGQ, spanning amino acids 67–71 in hARF1. It is identical to the cognate sequence in the alpha subunits of the heterotrimeric G proteins but differs from those of other small GTPases [58]. Similarly, the TCAT sequence (amino acids 158–161 in hARF 1) is identical to that of the corresponding region in most of the heterotrimeric G proteins. In ras and other small GTPases, this sequence is SAK (amino acids 145–147 in c-Ha-ras) [58]. The consensus sequence of the region thought to participate in guanine ring binding is

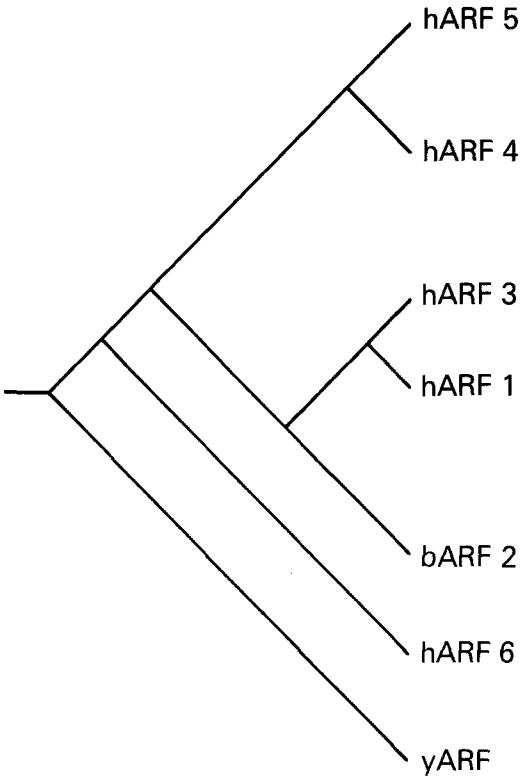

Fig. 3. Phylogenetic analysis of ARF cDNA sequences. Coding region nucleotide sequences of hARFs 1, 3 to 6, bARF 2, and yARF (yARF1) were analyzed using DNADIST and KITSCH programs to determine evolutionary relationships. Branches indicate points of evolutionary divergence. Data are from Ref. 48.

NKQD (amino acids 126–129 in hARF 1).

Like G_o and G_i, many of the ARFs undergo N-terminal modification by myristoyl CoA:protein N-myristoyltransferase (NMT) [63–65]. Each of the ARFs contains a glycine in the first position, following the initiating methionine, which is the required substrate for NMT, although neighboring amino acids also influence substrate suitability [66]. N-terminal myristoylation is necessary for ARF to associate with Golgi membranes [63] where it functions in protein trafficking via vesicular transport. In contrast, ras proteins undergo carboxyl terminal processing including farnesylation, carboxy methylation, and palmitoylation which is similarly required for membrane association. Thus, ARF appears to have more in common with G protein alpha subunits than with other small GTPases when comparing GTP-binding consensus sequences and post-translational modification.

ARF structure and function analysis has focused on the amino terminus since it is the site of covalent lipid modification. CD spectral studies revealed that a peptide composed of the first 16 amino acids of ARF 1 (excluding the initial methionine) would likely form an alpha helix in a hydrophobic but not an aqueous environment [67]. Myristoylation of the amino-terminal glycine of the peptide stabilized the α helical structure even in an aqueous solution. Furthermore, an ARF deletion mutant, lacking the amino-terminal 17 residues ([Δ1–17]mARF1p) retained the ability to bind GTPγS but no longer required DMPC/cholate to do so [67]. Amino-terminal α helix formation may, therefore, influence the binding or release of guanine nucleotides by ARF. To what extent the myristoylation requirement for membrane association [63] reflects the need for α helix formation is unknown.

ARD 1 is a 64-kDa protein, recently identified by cDNA cloning whose carboxyl terminal ~18 kDa is highly homologous to known mammalian ARFs but lacks sequence corresponding to the amino-terminal 15 amino acids [68]. Although previous studies had suggested that the amino terminus is critical for ARF activity [67], ARD 1 stimulated

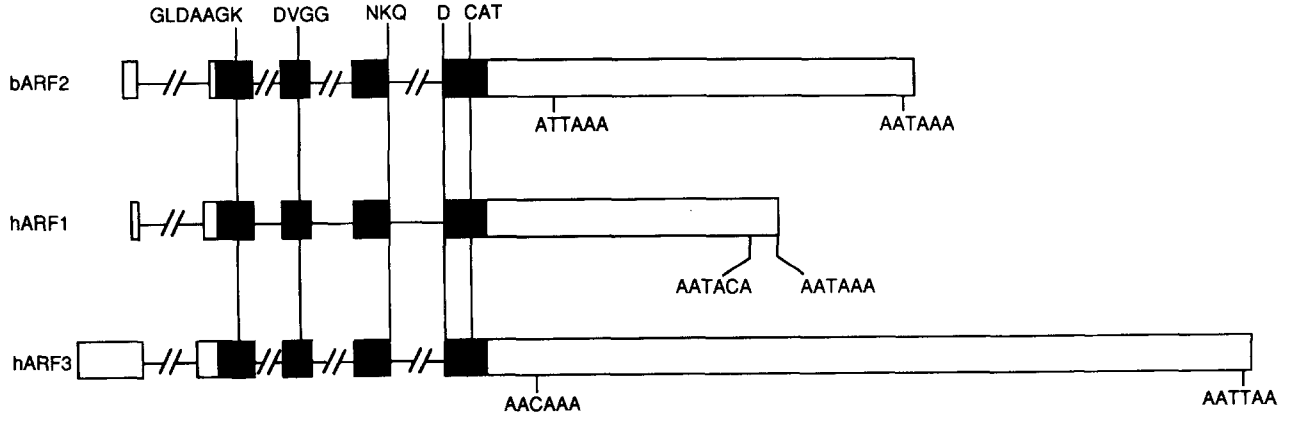

Fig. 4. Mammalian Class I ARF gene structures. Bovine ARF 2 (bARF2) [56], and human ARFs 1 and 3 (hARF1, 3) [54, 55] gene structures are compared. Exons are represented by boxes with coding regions shaded and untranslated regions open, introns by horizontal lines. Consensus sequences for GTP binding and hydrolysis are shown with locations indicated by vertical lines. Potential polyadenylation signal sequences and locations are indicated below each gene. Data are from Ref. 56.

cholera toxin ADP-ribosyltransferase activity in a GTP-dependent fashion in the presence of Tween 20 but not other lipids and detergents including DMPC/cholate. These studies clearly indicate that the amino terminal sequence is not responsible for ARF activity, although it may be involved with phospholipid interaction.

ARF's role in vesicular transport

Accumulating evidence has implicated ARF as one of a growing number of GTPases for which a role in intracellular protein transport has been recognized. In *Saccharomyces cerevisiae*, disruption of the yARF 1 gene, which accounts for 90% of ARF protein, caused a defect in invertase secretion with intracellular accumulation of incompletely glycosylated invertase [69].

In *in vitro* studies ARF shifts between cytosolic and membrane compartments in a guanine nucleotide-dependent cycle. In the presence of nonhydrolyzable GTP analogues, cytosolic ARF associates with membranes or anionic phospholipid vesicles [70, 71]. In NRK cells, ARF bound to Golgi membranes in the presence of GTPγS, an action inhibited by prior treatment with brefeldin A [72]. Brefeldin A is a fungal metabolite that disrupts Golgi architecture and blocks secretion [73, 74].

Serafini *et al.* [75] have identified ARF as a coat protein, along with α-, β-, γ-, and δ-COPs, major constituents of the non-clathrin coat of Golgi-derived vesicles. ARF was found associated with coated vesicles, which originate from the budding surface of Golgi membranes, and not with uncoated vesicles, which had bound to, but not fused with, the target Golgi membranes. ARF was also required for the GTPγS-dependent binding of β-COP, and therefore, presumably, the coat protein complex, the 'coatomer' [76].

Golgi-localized proteins that regulate the activation state of ARF and thus, its subcellular localization have been detected. A protein that enhanced the exchange of GDP bound to ARF with cytosolic GTP [77, 78] permitted ARF to associate with Golgi membranes. Rapid hydrolysis of the γ-phosphate of ARF-bound GTP occurred after the exchange reaction followed by release of ARF to the cytosolic fraction [77]. Subsequent studies revealed that individual ARFs differ in their subcellular localization and the conditions that promote membrane association [79, 80]. In addition, differences in the sensitivity to brefeldin A among ARF proteins have been reported [80].

The evidence supports a model for ARF's role in vesicular transport as proposed by Serafini *et al.* [75] in which ARF binds to the surface of Golgi membranes through interaction with a Golgi-localized exchange protein. ARF-GTP is generated which is competent to associate with membrane lipids. Coat assembly and budding ensues until destabilization of the vesicle coat is triggered by the formation of ARF-GDP by an ARF-specific GTPase-activating protein (GAP) at the target membrane. Components of the vesicle coat and ARF-GDP are released and recycled. ARF has also been implicated in other events involving vesicular fusion including endoplasmic reticulum to cis-Golgi transport [81], endosome fusion during endocytosis [82], and nuclear vesicle fusion following mitosis [83]. Specificity in targeting in each of these pathways may rely on individual ARFs interacting with specifically localized exchange proteins and GAPs.

Summary

ARF was discovered because of its role as a cofactor for cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$. ARF itself was found to be a GTP-binding protein and a direct allosteric activator of all of the toxin's catalytic activities. The ARFs are a family of highly homologous proteins that are conserved both structurally and functionally across distantly related eukaryotes and are distinct from other GTP-binding proteins. Biochemical features which distinguish ARFs include a relative dependence on phospholipids for GTP binding and activity, little or no intrinsic GTPase activity, and amino-terminal modification by N-myristoyltransferase. *In vivo*, ARF has been implicated in the regulation of vesicular transport through a guanine nucleotide-dependent cycle of association with and release from Golgi membranes. ARF has been identified as a coat protein and ARF-specific regulatory proteins which catalyze guanine nucleotide exchange and accelerate GTPase activity have been detected on Golgi membranes. Understanding ARF function and regulation will be important in elucidating mechanisms of vesicular transport.

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References

1. Gill DM: The arrangement of subunits in cholera toxin. Biochemistry 15: 1242–1248, 1976
2. Gill DM: Mechanism of action of cholera toxin. Adv Cyclic Nucleotide Res 8: 85–118, 1977
3. Moss J, Vaughan M: Participation of guanine nucleotide-binding protein cascade in activation of adenylyl cyclase by cholera toxin (choleragen). In: R. Iyengar and L. Birnbaumer (eds). G Proteins, Academic Press, San Diego, CA, 1990, pp 179–200
4. Mekalanos JJ, Collier RJ, Romig WR: Enzymic activity of cholera toxin. II. Relationships to proteolytic processing, disulfide bond reduction, and subunit composition. J Biol Chem 254: 5855–5861, 1979

5. Moss J, Stanley SJ, Lin MC: NAD glycohydrolase and ADP-ribosyltransferase activities are intrinsic to the A_1 peptide of choleragen. J Biol Chem 254: 11993–11996, 1979
6. Moss J, Manganiello VC, Vaughan M: Hydrolysis of nicotinamide adenine dinucleotide by choleragen and its A protomer: possible role in the activation of adenylate cyclase. Proc Natl Acad Sci USA 73: 4424–4427, 1976
7. Moss J, Vaughan M: Mechanism of action of choleragen. Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. J Biol Chem 252: 2455–2457, 1977
8. Mekalanos JJ, Collier RJ, Romig WR: Enzyme activity of cholera toxin. I. New methods of assay and the mechanism of ADP-ribosyl transfer. J Biol Chem 254: 5849–5854, 1979
9. Moss J, Vaughan M: Isolation of an avian erythrocyte protein possessing ADP-ribosyltransferase activity and capable of activating adenylate cyclase. Proc Natl Acad Sci USA 75: 3621–3624, 1978
10. Moss J, Stanley SJ, Watkins PA, Vaughan M: ADP-ribosyltransferase activity of mono- and multi-(ADP-ribosylated) choleragen. J Biol Chem 255: 7835–7837, 1980
11. Trepel JB, Chuang D-M, Neff NH: Transfer of ADP-ribose from NAD to choleragen: A subunit acts as a catalyst and acceptor protein. Proc Natl Acad Sci USA 74: 5440–5442, 1977
12. Cassel D, Pfeuffer T: Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. Proc Natl Acad Sci USA 75: 2669–2673, 1978
13. Gill DM, Meren R: ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc Natl Acad Sci USA 75: 3050–3054, 1978
14. Johnson GL, Kaslow HR, Bourne HR: Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. J Biol Chem 253: 7120–7123, 1978
15. Northup JK, Sternweis PC, Smigel MD, Schleifer LS, Ross EM, Gilman AG: Purification of the regulatory component of adenylate cyclase. Proc Natl Acad Sci USA 77: 6516–6520, 1980
16. Finkelstein RA: Cholera. CRC Crit Rev Microbiol 2: 553–623, 1973
17. Kelley MT: Cholera: a worldwide perspective. Pediat Infect Dis 5: S101–S105, 1986
18. Gill DM: Involvement of nicotinamide adenine dinucleotide in the action of cholera toxin *in vitro*. Proc Natl Acad Sci USA 72: 2064–2068, 1975
19. Enomoto K, Gill DM: Requirement for guanosine triphosphate in the activation of adenylate cyclase by cholera toxin. J Supramol Struct 10: 51–60, 1979
20. Moss J, Vaughan M: Choleragen activation of solubilized adenylate cyclase: requirement for GTP and protein activator for demonstration of enzymatic activity. Proc Natl Acad Sci USA 74: 4396–4400, 1977
21. Lin MC, Welton AF, Berman MF: Essential role of GTP in the expression of adenylate cyclase activity after cholera toxin treatment. J Cyclic Nucleotide Res 4: 159–168, 1978
22. Nakaya S, Moss J, Vaughan M: Effects of nucleoside triphosphates on choleragen-activated brain adenylate cyclase. Biochemistry 19: 4871–4874, 1980
23. Enomoto K, Gill DM: Cholera toxin activation of adenylate cyclase. Roles of nucleoside triphosphates and a macromolecular factor in the ADP-ribosylation of the GTP-dependent regulatory component. J Biol Chem 255: 1252–1258, 1980
24. Gill DM, Meren R: A secondary guanyl nucleotide-binding site associated with adenylate cyclase. Distinct nucleotides activate adenylate cyclase and permit ADP-ribosylation by cholera toxin. J Biol Chem 258: 11908–11914, 1983
25. LeVine H III, Cuatrecasas P: Activation of pigeon erythrocyte adenylate cyclase by cholera toxin. Partial purification of an essential macromolecular factor from horse erythrocyte cytosol. Biochim Biophys Acta 672: 248–261, 1981
26. Schleifer LS, Kahn RA, Hanski E, Northup JK, Sternweis PC, Gilman AG: Requirements for cholera toxin-dependent ADP-ribosylation of the purified regulatory component of adenylate cyclase. J Biol Chem 257: 20–23, 1982
27. Pinkett MO, Anderson WB: Plasma membrane-associated component(s) that confer(s) cholera toxin sensitivity to adenylate cyclase. Biochim Biophys Acta 714: 337–343, 1982
28. Gill DM, Woolkalis MJ: [^{32}P]ADP-ribosylation of proteins catalyzed by cholera toxin and related heat-labile enterotoxins. Methods Enzymol 165: 235–245, 1988
29. Schmidt GJ, Huber LJ, Weiter JJ: A-protein catalyzes the ADP-ribosylation of G-protein from cow rod outer segments. J Biol Chem 262: 14333–14336, 1987
30. Gill DM, Coburn J: ADP-ribosylation by cholera toxin: functional analysis of a cellular system that stimulates the enzymatic activity of cholera toxin fragment A_1. Biochemistry 26: 6364–6371, 1987
31. Woolkalis M, Gill DM, Coburn J: Assay and purification of cytosolic factor required for cholera toxin activity. Methods Enzymol 165: 246–249, 1988
32. Kahn RA, Gilman AG: Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. J Biol Chem 259: 6228–6234, 1984
33. Kahn RA, Gilman AG: The protein cofactor necessary for ADP-ribosylation of G_s by cholera toxin is itself a GTP binding protein. J Biol Chem 261: 7906–7911, 1986
34. Tsai S-C, Noda M, Adamik R, Moss J, Vaughan M: Enhancement of choleragen ADP-ribosyltransferase activities by guanyl nucleotides and a 19-kDa membrane protein. Proc Natl Acad Sci USA 84: 5139–5142, 1987
35. Tsai S-C, Noda M, Adamik R, Chang PP, Chen H-C, Moss J, Vaughan M: Stimulation of choleragen enzymatic activities by GTP and two soluble proteins purified from bovine brain. J Biol Chem 263: 1768–1772, 1988
36. Noda M, Tsai S-C, Adamik R, Moss J, Vaughan M: Mechanism of cholera toxin activation by a guanine nucleotide-dependent 19 kDa protein. Biochim Biophys Acta 1034: 195–199, 1990
37. Bobak DA, Blizotes MM, Noda M, Tsai S-C, Adamik R, Moss J: Mechanism of activation of cholera toxin by ADP-ribosylation factor (ARF): Both low- and high-affinity interactions of ARF with guanine nucleotides promote toxin activation. Biochemistry 29: 855–861, 1990
38. Tsai S-C, Adamik R, Moss J, Vaughan M: Guanine nucleotide dependent formation of a complex between choleragen (cholera toxin) A subunit and bovine brain ADP-ribosylation factor. Biochemistry 30: 3697–3703, 1991
39. Weiss O, Holden J, Rulka C, Kahn RA: Nucleotide binding and cofactor activities of purified bovine brain and bacterially expressed ADP-ribosylation factor. J Biol Chem 264: 21066–21072, 1989
40. Kahn RA, Kern FG, Clark J, Gelmann EP, Rulka C: Human ADP-ribosylation factors. A functionally conserved family of GTP-binding proteins. J Biol Chem 266: 2606–2614, 1991
41. Price SR, Welsh CF, Haun RS, Stanley SJ, Moss J, Vaughan M: Effects of phospholipid and GTP on recombinant ADP-ribosylation factors (ARFs). Molecular basis for differences in requirements for activity of mammalian ARFs. J Biol Chem 267: 17766–17772, 1992
42. Tsai S-C, Adamik R, Tsuchiya M, Chang PP, Moss J, Vaughan M: Differential expression during development of ADP-ribosylation factors, 20-kDa guanine nucleotide-binding protein activators of cholera toxin. J Biol Chem 266: 8213–8219, 1991
43. Mishima K, Price SR, Nightingale MS, Kousvelari E, Moss J, Vaughan M: Regulation of ADP-ribosylation factor (ARF) expression. Cross-species conservation of the developmental and tissue-specific alterna-

tive polyadenylation of ARF 4 mRNA. J Biol Chem 267: 24109–24116, 1992
44. Sewell JL, Kahn RA: Sequences of the bovine and yeast ADP-ribosylation factor and comparison to other GTP-binding proteins. Proc Natl Acad Sci USA 85: 4620–4624, 1988
45. Bobak DA, Nightingale MS, Murtagh JJ, Price SR, Moss J, Vaughan M: Molecular cloning, characterization, and expression of human ADP-ribosylation factors: two guanine nucleotide-dependent activators of cholera toxin. Proc Natl Acad Sci USA 86: 6101–6105, 1989
46. Price SR, Nightingale MS, Tsai S-C, Williamson KC, Adamik R, Chen H-C, Moss J, Vaughan M: Guanine nucleotide-binding proteins that enhance choleragen ADP-ribosyltransferase activity: nucleotide and deduced amino acid sequence of an ADP-ribosylation factor cDNA. Proc Natl Acad Sci USA 85: 5488–5491, 1988
47. Monaco L, Murtagh JJ, Newman KB, Tsai S-C, Moss J, Vaughan M: Selective amplification of an mRNA and related pseudogene for a human ADP-ribosylation factor, a guanine nucleotide-dependent protein activator of cholera toxin. Proc Natl Acad Sci USA 87: 2206–2210, 1990
48. Tsuchiya M, Price SR, Tsai S-C, Moss J, Vaughan M: Molecular identification of ADP-ribosylation factor mRNAs and their expression in mammalian cells. J Biol Chem 266: 2772–2777, 1991
49. Peng Z, Calvert I, Clark J, Helman L, Kahn R, Kung H-F: Molecular cloning, sequence analysis and mRNA expression of human ADP-ribosylation factor. Biofactors 2: 45–49, 1989
50. Stearns T, Kahn RA, Botstein D, Hoyt MA: ADP-ribosylation factor is an essential protein in *Saccharomyces cerevisiae* and is encoded by two genes. Mol Cell Biol 10: 6690–6699, 1990
51. Murtagh JJ Jr, Lee F-JS, Deak P, Hall LM, Monaco L, Lee C-M, Stevens LA, Moss J, Vaughan M: Molecular characterization of a conserved, guanine nucleotide-dependent ADP-ribosylation factor in *Drosophila melanogaster*. Biochemistry, 31: 6011–6018, 1993
52. Murtagh JJ Jr, Mowatt MR, Lee C-M, Lee F-JS, Mishima K, Nash TE, Moss J, Vaughan M: Guanine nucleotide-binding proteins in the intestinal parasite *Giardia lamblia*. Isolation of a gene encoding an ~20 kDa ADP-ribosylation factor. J Biol Chem 267: 9654–9662, 1992
53. Tsuchiya M, Price SR, Nightingale MS, Moss J, Vaughan M: Tissues and species distribution of mRNA encoding two ADP-ribosylation factors, 20-kDa guanine nucleotide binding proteins. Biochemistry 28: 9668–9673, 1989
54. Tsai S-C, Haun RS, Tsuchiya M, Moss J, Vaughan M: Isolation and characterization of the human gene for ADP-ribosylation factor 3, a 20-kDa guanine nucleotide-binding protein activator of cholera toxin. J Biol Chem 266: 23053–23059, 1991
55. Lee C-M, Haun RS, Tsai S-C, Moss J, Vaughan M: Characterization of the human gene encoding ADP-ribosylation factor 1, a guanine nucleotide-binding activator of cholera toxin. J Biol Chem 267: 9028–9034, 1992
56. Serventi IM, Cavanaugh E, Moss J, Vaughan M: Characterization of the gene for ADP-ribosylation factor (ARF) 2, developmentally regulated, selectively expressed member of the ARF family of ~20-kDa guanine nucleotide-binding proteins. J Biol Chem 268: 4863–4864, 1993
57. Haun RS, Moss J, Vaughan M: Characterization of the human ADP-ribosylation factor 3 promoter. Transcriptional regulation of a TATA-less promoter. J Biol Chem 268: 8793–8800, 1993
58. Price SR, Barber A, Moss J: Structure-function relationships of guanine nucleotide-binding proteins. In: J. Moss and M. Vaughan (eds). ADP-ribosylating Toxins and G Proteins: Insights Into Signal Transduction, American Society for Microbiology, Washington, DC, 1990, pp 397–424
59. Gibbs JB, Sigal IS, Poe M, Scolnick EM: Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc Natl Acad Sci USA 81: 5704–5708, 1984
60. McGrath JP, Capon DJ, Goeddel DV, Levinson AD: Comparative biochemical properties of normal and activated human *ras* p21 protein. Nature 310: 644–649, 1984
61. Sweet RW, Yokoyama S, Kamata T, Feramisco JR, Rosenberg M, Gross M: The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature 311: 273–275, 1984
62. Tamkun JW, Kahn RA, Kissinger M, Brizuela BJ, Rulka C, Scott MP, Kennison JA: The arflike gene encodes an essential GTP-binding protein in *Drosophila*. Proc Natl Acad Sci USA 88: 3120–3124, 1991
63. Haun RS, Tsai S-C, Adamik R, Moss J, Vaughan M: Effect of myristoylation on GTP-dependent binding of ADP-ribosylation factor to Golgi. J Biol Chem 268: 7064–7068, 1993
64. Kahn RA, Goddard C, Newkirk M: Chemical and immunological characterization of the 21-kDa ADP-ribosylation factor of adenylate cyclase. J Biol Chem 263: 8282–8287, 1988
65. Kunz BC, Muczynski KA, Welsh CF, Stanley SJ, Tsai S-C, Adamik R, Chang PP, Moss J, Vaughan M: Characterization of recombinant and endogenous ADP-ribosylation factors synthesized in Sf9 insect cells. Biochemistry 32: 6643–6648, 1993
66. Towler DA, Adams SP, Eubanks SR, Towery DS, Jackson-Machelski E, Glaser L, Gordon JI: Purification and characterization of yeast myristoyl CoA:protein N-myristoyltransferase. Proc Natl Acad Sci USA 84: 2708–2712, 1987
67. Kahn RA, Randazzo P, Serafini T, Weiss O, Rulka C, Clark J, Amherdt M, Roller P, Orci L, Rothman JE: The amino terminus of ADP-ribosylation factor (ARF) is a critical determinant of ARF activities and is a potent and specific inhibitor of protein transport. J Biol Chem 267: 13039–13046, 1992
68. Mishima K, Tsuchiya M, Nightingale MS, Moss J, Vaughan M: ARD 1, a 64-kDa guanine nucleotide-binding protein with a carboxyl-terminal ADP-ribosylation factor domain. J Biol Chem 268: 8801–8807, 1993
69. Stearns T, Willingham MC, Botstein D, Kahn RA: ADP-ribosylation factor is functionally and physically associated with the Golgi complex. Proc Natl Acad Sci USA 87: 1238–1242, 1990
70. Regazzi R, Ullrich S, Kahn RA, Wollheim CB: Redistribution of ADP-ribosylation factor during stimulation of permeabilized cells with GTP analogues. Biochem J 275: 639–644, 1991
71. Walker MW, Bobak DA, Tsai S-C, Moss J, Vaughan M: GTP but not GDP analogues promote association of ADP-ribosylation factors, 20-kDa protein activators of cholera toxin, with phospholipids and PC-12 cell membranes. J Biol Chem 267: 3230–3235, 1992
72. Donaldson JG, Kahn RA, Lippincott-Schwartz J, Klausner RD: Binding of ARF and β-COP to Golgi membranes: Possible regulation by a trimeric G protein. Science 254: 1197–1199, 1991
73. Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD: Rapid redistribution of Golgi proteins into the ER in cells treated with Brefeldin A: evidence for membrane cycling from Golgi to ER. Cell 56: 801–813, 1989
74. Lippincott-Schwartz J, Donaldson JG, Schweizer A, Berger EG, Hauri H-P, Yuan LC, Klausner RD: Microtubule-dependent retrograde transport of proteins into the ER in the presence of Brefeldin A suggests an ER recycling pathway. Cell 60: 821–836, 1990
75. Serafini T, Orci L, Amherdt M, Brunner M, Kahn RA, Rothman JE: ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. Cell 67: 239–253, 1991
76. Donaldson JG, Cassel D, Kahn RA, Klausner RD: ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatomer protein β-COP to Golgi membranes. Proc Natl Acad Sci USA 89: 6408–6412, 1992
77. Donaldson JG, Finazzi D, Klausner RD: Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF pro-

tein. Nature 360: 350–352, 1992
78. Helms JB, Rothman JE: Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. Nature 360: 352–354, 1992
79. Tsai S-C, Adamik R, Haun RS, Moss J, Vaughan M: Differential interaction of ADP-ribosylation factors 1, 3, and 5 with rat brain Golgi membranes. Proc Natl Acad Sci USA 89: 9272–9276, 1992
80. Tsai S-C, Adamik R, Haun RS, Moss J, Vaughan M: Effects of brefeldin A and accessory proteins on association of ADP-ribosylation factors 1, 3, and 5 with Golgi. J Biol Chem 268: 10820–10825, 1993
81. Balch WE, Kahn RA, Schwaninger R: ADP-ribosylation factor is required for vesicular trafficking between the endoplasmic reticulum and the *cis*-Golgi compartments. J Biol Chem 267: 13053–13061, 1992
82. Lenhard JM, Kahn RA, Stahl PD: Evidence for ADP-ribosylation factor (ARF) as a regulator of *in vitro* endosome-endosome fusion. J Biol Chem 267: 13047–13052, 1992
83. Boman AL, Taylor TC, Melançon P, Wilson KL: A role for ADP-ribosylation factor in nuclear vesicle dynamics. Nature 358: 512–514, 1992